- ANNUAL PROGRESS REPORT 1966

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Title of Project: Integrated Research and Training in Space-Molecular Biology

Period of Project: April 1, 1966 through December 31, 1966

Institution: The University of Chicago

Principal Investigator: Humberto Fernández-Morán, M.D., Ph.D.
Professor of Biophysics
Department of Biophysics

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INDEX

Summary of Research Accomplishments for 1966.....Page ii-iv

I. Specific Research Program

A. Report of Participation in Luster Sounding Rocket Experiment.....Page 1-8

B. Correlated Electron Microscopic and Electron Diffraction Studies of Certain Meteorites and Pre-cambrian Systems.....Page 9

C. Experimental Program in Electron Microscopy with Superconducting Lenses.....Page 10-12

D. Development of Improved Instrumentation and Preparation Techniques.....Page 13-16

E. Studies on the Subunit Structure of Membranes, including Mitochondria, Nerve Membranes, Photoreceptors: Retinal Rod Outer Segments, Multienzyme Complexes, Fraction-I Protein, and RNA-polymerase.....Page 17-22

II. Organization and Operation of Special Electron Microscope Laboratories.....Page 23-24

III. Training Program.....Page 25-26

IV. List of Publications.....Page 27-29

NASA GRANT NsG 441-63
ANNUAL PROGRESS REPORT 1966

SUMMARY OF RESEARCH ACCOMPLISHMENTS FOR 1966

During the past year significant progress has been made in the development and systematic application of improved instrumentation and preparation techniques for high resolution electron microscopy of membranes and related biological systems. This included studies by combined high resolution electron microscopy and electron diffraction techniques of specimens of meteor stream particles and other extra-terrestrial components collected by the Luster Sounding Rocket Experiments from the outer fringe of the earth's atmosphere during the Orionid Meteor Shower, October 22, 1966 (White Sands Missile Range).

The following accomplishments can be singled out as highlights of our research project: (a) Further research and development work has been carried out on improved methodology for collecting and identifying extra-terrestrial particles. In this connection, promising exploratory experiments are currently in progress for in-flight shadow casting using an exploding wire technique. These experiments indicate that it is in principle possible to coat incoming particles with a metal shadowing layer at the instant of exposing the collecting surfaces of the sampling instrument. We could hereby actually obtain a reliable submicroscopic snapshot of the particle, including a three-dimensional molecular replica for subsequent electron microscopic examination. This procedure would be of particular importance in labelling the particle clearly as extra-terrestrial and thus distinguishing it from any other contaminating material. It is believed that these and related approaches will become essential for all future experiments of this type in view of the extra-ordinarily complex and manifold sources of contamination which make the interpretation of present results so uncertain.

(b) With improved point cathode sources and electron gun design and specially stabilized power supplies operating under the optimum conditions provided in our new electron microscope facility, we have been able to reproducibly achieve point resolutions of 2 to 3Å. This represents the best consistently attained performance in approaching the theoretical resolution limit of transmission electron microscopes.

(c) Thanks to these methodological advances, it has now been possible for the first time to visualize directly the hexagonal cells of single-crystal graphite containing only 10 carbon atoms. This work was carried out in our laboratories confirming and extending parallel studies by R. Heidenreich of the Bell Telephone Laboratories. Demonstration of this capability of point-to-point resolution

in the range of interatomic distances was generally regarded as a significant contribution singled out among the advances made in physics and chemistry during the past year.

(d) Extension of this work and the enhanced capability of interpreting the phase contrast images now make it appear feasible to record detail of molecular and pauciatomic dimensions in biological systems. As shown in the attached micrographs we have recently succeeded in obtaining high resolution electron phase contrast images of single molecules of copper phthalocyanine, which can be correlated with corresponding diagrams of atomic positions as deduced from x-ray diffraction studies.

(e) Improved preparation techniques and instrumentation have been systematically applied to the study of biological systems under a wide variety of experimental conditions. Useful resolutions of 6 to 15Å were consistently attained, corresponding in many cases to direct visualization of quaternary or tertiary structures in biological membranes and derivatives, multienzyme complexes, hemocyanin and apohemocyanin, catalase and RNA polymerase, and DNA and RNA systems.

(f) The use of new types of phase contrast apertures has made it possible to obtain high resolution and enhanced contrast in stained and unstained biological specimens under conditions which considerably reduce radiation damage. Therefore, further improvements in low temperature preparation techniques of native biological specimens appear to be particularly promising.

These results bring us closer to the ultimate goal of high resolution electron microscopy, namely, direct readout of molecular structure. This represents one of the greatest potentials of electron microscopy's key role in the study of biomolecular organization which is a central unifying discipline of the natural sciences and of biomedical research in general.

(g) Our work with superconducting lenses represents another significant approach to the high resolution electron microscopic examination of biological specimens in the frozen hydrated state under ideal low temperature conditions of minimized specimen contamination, radiation damage, and thermal noise.

NASA GRANT NsG 441-63
ANNUAL PROGRESS REPORT 1966

We have recorded, for the first time, electron micrographs of biological specimens at 4.2°K. Resolutions of 10-20Å were reproducibly attained, particularly in specimens of catalase crystals embedded in thin layers of heavy metals. The pictures exhibit remarkable contrast and anomalous transparency phenomena which, because of its association with considerably reduced radiation damage at low temperatures, promises to greatly facilitate examination of biological specimens at liquid helium temperatures.

This work resulted from the use of our cryo-electron microscope, equipped with superconducting objective lens in a specially designed dewar with superconducting stigmators, persistent current switches and improved superconducting current control devices. The unique combination of high magnetic fields, liquid helium temperatures and high electron optical magnifications has enabled us to make preliminary observations on characteristic electron optical phenomena associated with trapped fluxes in thin superconducting films.

These results were presented in a keynote address before the Sixth International Congress for Electron Microscopy at Kyoto, Japan, August 28 to September 4, 1966.

International evaluation, as expressed in particular by Prof. Dupouy of the Laboratoire d'Optique du C.N.R.S. at Toulouse, was that our pioneering work has clearly established the value of superconducting solenoid lenses for electron microscopy, especially because of their superstability, the unprecedented high fields, and the possibility of now being able to consider other types of magnetic field configuration for obtaining much higher performance in high resolution electron microscopes.

Prof. Dupouy claimed as a noteworthy achievement the fact that since the first successful imaging experiments were carried out barely 2 1/2 years ago, we have been able to implement a comprehensive research program, including the development and testing of many difficult components and solving other related methodological and conceptual problems.

Our work was also confirmed by Dr. Laberrigue, College de France in Paris, who stated that a new chapter has been opened up in electron optics with superconducting lenses which should prove to be of key significance for high voltage electron microscopy.

NASA GRANT NsG 441-63
ANNUAL PROGRESS REPORT 1966

Our activities during the past year have been carried out according to the program set forth in our renewal proposal for Grant NsG 441-63 (April 1, 1966 to December 31, 1966) and include:

I. Specific Research Program.

A. We have continued participation in the Luster Sounding Rocket Experiment.

1. Introduction

A Luster micrometeorite sampling instrument was launched October 22, 1966, at White Sands Missile Range, New Mexico, and was successfully recovered. The payload was launched on an Aerobee 150 sounding rocket near the peak of the Orionid meteor shower.

The Luster payload has been described in detail by Blanchard and Farlow (Contamination Control, 5, 22, 1966). The instrument carried 12 vacuum sealed modules on three deployable arms to a maximum altitude of about 145 km. Above 60 km. the arms extended, and pans containing $1m^2$ of sampling surfaces were exposed upward for about 200 secs. The mating covers remained fixed to the central structure and exposed another $1m^2$ outward. The instrument resealed itself prior to atmospheric reentry, and the modules became vacuum sealed again as the outside pressure rose. Parachute descent completed the recovery. Instruments on board and on the ground revealed that the vacuum was retained throughout the flight and that proper opening and closing occurred. The flight samples were shipped in vacuum containers to the Electron Microscope Laboratory of the University of Chicago, where they were opened in clean room conditions.

2. Material and Methods

Three nearly identical modules were prepared. One, designated Whitesands, was exposed to clean room environment at White Sands Missile Range for 1 hour on October 21, 1966, during loading of Luster 2 payload. A second, the Ames capsule, was exposed at Ames Research Center during the packing of the on-flight specimen. It was put into the modules on

laminar flow benches and sealed in identical fashion. The third was the on-flight Luster capsule.

All substrates were prepared in the clean room facilities of the Electron Microscope Laboratory at the University of Chicago. These laboratories have a special air-conditioning system designed to maintain relative humidity not exceeding 50%, and are provided with Cambridge absolute filters excluding particles greater than 0.1 microns.

The thin film substrates were made by evaporation of carbon or carbon-SiO in an ultrahigh vacuum (10^{-8} torr) produced by a Varian vacuum unit, consisting of an ion pump and Linde molecular sorption forpump with liquid nitrogen cold trap. This eliminated any contamination with oil vapors. The films were stripped off from freshly cleaved mica onto quartz distilled, ultra-filtered water.

The platinum specimen holders were of three types:

a. slit holder--1mm x 0.1mm
b. 7 hole--50μ diameter
c. single hole--50μ diameter

All of the platinum grids and stainless steel slides were sonicated on a Sonogen Automatic Cleaner using Millipore filter distilled petroleum ether, acetone, and ethel alcohol. Ultra-filtered quartz-distilled water was used at all times. The platinum grids, which are chemically inert, were cleaned in this way and then heated to incandescence in an ultrahigh vacuum (2×10^{-8} torr).

Each container contained the following substrates:

(1) Freshly cleaved mica on stainless steel slides

(a) The mica strip was exposed onto precleaned stainless steel slides using Walsco Epoxy Cement.

(b) The slides were then heated on hot plate to harden cement.

(c) The upper layers of the mica were then cleaved off, leaving an atomically smooth surface.

(d) The slides were shadowed at an 90 degree angle with carbon from a height of 14cm. at a vacuum of approximately 5×10^{-7} torr.

(e) They were then stored in petri dishes in a dessicator over silica gel.

(2) Lucite slides

(a) The lucite was pre-cleaned with methanol.

(b) The slides were placed in the Varian and shadowed as follows: SiO--height 9cm, angle 30^{o}, vacuum 8×10^{-7}; Carbon--height 14cm, angle 90^{o}, vacuum 6×10^{-7} torr.

(3) Platinum specimen holders

(a) 0.3% formvar fenestrated film covered with thin carbon film, evaporated at 2.5×10^{-7} torr. and height 14cm.

(b) 0.3% formvar fenestrated film covered with SiO and carbon (vacuum 9×10^{-7} torr. SiO height 9cm; carbon height 14cm, both perpendicular).

(c) 0.3% formvar film covered with SiO and carbon.

(d) 0.3% formvar film and thick carbon. These were made by coating mica with carbon (vacuum 2×10^{-7} torr, height 14cm.) then dipping into formvar, floating onto water and placing on grids.

(e) 0.3% formvar film and carbon film.

(f) 0.3% formvar film and medium carbon film.

The substrates were photographed using the Nikon Shadowgraph and Gravure film (ASA 12) and then distributed to the capsules as follows:

Luster

2 Lucite slides
1 Mica slide
1 stainless steel slide with 144 Pt. grids

Ames and Whitesands

1 Lucite slide
1 Mica slide
1 Stainless steel slide with 30 Pt. grids

The containers were then assembled and placed in the Varian and given a final carbon shadow. A medium carbon coat was given with the vacuum at 5×10^{-7} torr. in all three cases. The containers were then sealed and put under a vacuum of 1×10^{-6} torr.

The electron microscopy and selected area high resolution electron diffraction were carried out using a Siemens Elmiscope I microscope. With this instrument it was possible to use low intensity microbeam illumination and liquid nitrogen decontamination device to view particles that could otherwise have been volatilized by the beam. The microscope was carefully calibrated from 225X - 110,000X and compensated to within one ultrafine step (300Å).

3. Results

Examination of the controls for the Luster project is still in progress, but preliminary results show a general similarity to those reported previously. A complete report will be presented at a later date when more extensive results are available.

Mr. Lou Sherman, a graduate student in the Department of Biophysics and Miss D. Meddoff collaborated in the preparation and examination of the controls. Mr. Sherman personally carried the specimens to Ames Research Center and returned with them.

The examination by light microscopy, electron microscopy, and electron diffraction consisted of three parts: (a) study of grids left in EM Lab (b) Ames Control and (c) Whitesands Control. To date, some 60% of all the platinum grid controls have been examined in detail, using magnifications ranging from 225X - 80,000X. This has resulted in a catalogue of the type of terrestrial contaminants likely to be found on our substrates, and has enabled us to make some significant comparisons with the results from the Luster 1 experiments. It should also be of great help in enabling the Luster 2 findings to expand and refine the preliminary investigations.

The control substrates were found to be relatively clear and unbroken. The vast majority of contaminating particles were mica particles, which were introduced during the stripping of the films from the cleaved mica. These are easily recongnizable by their characteristic structure and electron diffraction patterns. At times, a very thin piece of mica yielded a characteristic single-crystal diffraction pattern. In three places, contaminating microorganisms such as bacteria were found, but this is rare. A few dust and dirt particles were found, but these were all in the 1 - 20μ range.

Quantitatively, the results are statistically significant when compared with certain findings from Luster 1. From over 1mm^2 of area (10^6 μ^2), the highest density of contamination found was 1 particle/30 square micron.

Typical values were one particle /600 square micron for some of the lab. controls, to one particle /10,000μ^2 for some of the slit grids on the Ames control. This should be contrasted to a density of some 50 particles /μ^2 in some areas of Luster 1. If we are to take this background noise as representative, we narrow down quite radically the possible origin of many of the high density areas on Luster 1. Either they have an extra-terrestrial origin, or they are contamination that occurred during the flight, possibly from the rocket itself.

The size differences are also of importance here. Almost all particles found in the controls, were in the micron range. The average of 122 particles measured from the Whitesands controls was 1.63μ and of 80 particles on the Ames control, 1.32μ. (These were measured on the Nikon Shadowgraph). Some of these particles did resemble the micron range particles found on Luster 1 in size and general morphology. However, they did not react in the same way under the electron beam bombardment, and so the Luster 1 findings can not be disregarded as yet.

On the other hand, no particles less than 500Å were found, except in one case which will be considered later. To be more specific, no particles in the 20 - 100Å range were seen, which should be contrasted with the numerous particles of this size found on Luster 1. The fact that particles of this size were normally found in areas of high particle density can lead to a hypothesis that these are fragments caused by particles impacting on the substrate. This is definitely worthy of further consideration.

In this respect, the controls showed no examples of fissures as were found on Luster 1. Since this type of tear in the film is never seen under other experimental conditions and are always associated with particles, they may indicate impact of a particle and consequent rupture of the film. This would also verify some of the findings of Hemenway and Soberman.

In one area, some 100Å particles were found. These were associated with some very thin particles of mica, and yielded a single-crystal diffraction pattern. These particles and the bigger aggregates with which they were associated did not resemble anything found on Luster 1, and their significance is uncertain. Morphologically, they resemble somewhat fine dust particles, but more work would need to be done to characterize their nature.

Some particles with a dense core and light coat were found which resemble closely those found on Luster 1. However, none were found associated with ruptures of the substrate, nor were they sensitive to the electron beam. Again, the significance of this result is not understood.

Finally, no particles resembling the electron sensitive amorphous material was found in any of the controls. If this material is organic material, it seems unlikely that it was caused by contamination during any of the steps involved in preparing the substrates. If it is contaminated, it must be from the rocket system. In this line, it would seem that model experiments, testing gaseous vapors from the rocket propulsion system, would be a great help in clearing this problem.

4. Summary

From this survey of the contaminants found on the controls of the Luster 2 experiments, we can see that the substrates are clean and unbroken. It has been shown that particle density and size may be very important in determination of the possible origin of the particles. Some control particles, though resembling morphologically Luster 1 particles, react differently under the electron beam, and, thus, should be composed of different material. This, then, would seem to be the next important step. Electron diffraction studies, electron micro-probe studies and chemical analysis are of the utmost importance. Chemical analysis and critical high resolution electron microscopy and electron diffraction studies of the particles would seem to be essential before assigning an extra-terrestrial origin to a particle. That is why the highest level of credibility would seem to apply to the 20 - 100Å amorphous (organic?) material. If a chemical analysis could show this to be different than anything found on controls (and in the rocket's fuel) it would be a significant discovery. The

extraction and electron diffraction experiments could be carried out on Luster 2 to try to confirm the preliminary results.

Yet to be done are the mica and lucite slides. The main benefit of these, of course, would be particle impact studies, to see if we are dealing with particles having a velocity high enough to embed itself in the material. This would be another step to show that we are dealing with extra-terrestrial material.

5. Outlook

Promising exploratory experiments are currently in progress for in-flight shadow casting using an exploding wire technique. These experiments indicate that it is in principle possible to coat incoming particles with a metal shadowing layer at the instant of exposing the collecting surfaces of the sampling instrument. We could hereby actually obtain a reliable submicroscopic snapshot of the particle, including a three-dimensional molecular replica for subsequent electron microscopic examination. This procedure would be of particular importance in labelling the particle clearly as extra-terrestrial and thus distinguishing it from any other contaminating material. It is believed that these and related approaches will become essential for all future experiments of this type in view of the extra-ordinarily complex and manifold sources of contamination which make the interpretation of present results so uncertain.

B. Continuation of Correlated Electron Microscopic and Electron Diffraction Studies of Certain Meteorites and of Pre-Cambrian Organized Systems.

1. Investigation of Orgueil Carbonaceous Chondrite.

We have been arranging to continue electron microscopic and electron diffraction studies of certain meteorites (Orgueil carbonaceous chondrite) carried out with Dr. Edward Anders and Dr. Frank Fitch of the University of Chicago. Preliminary experiments indicate that the composition and structural relationships of its constituents can be determined by the high resolving power of the electron microscope. Various preparation techniques are being applied under carefully controlled conditions. These techniques include ultrathin sectioning with a diamond knife, mechanical and selective chemical dissociation, followed by density gradient separation, negative staining, shadow-casting, etc. The ultrastructural data will be correlated with parallel chemical studies of organic constituents and with the results of selected area electron diffraction analysis.

2. Investigation of Pre-cambrian Rocks of Gunflint Chert Formation of Southern Ontario, Canada.

We are also arranging to continue electron microscopical studies of Pre-cambrian organized systems. Preliminary investigations of nonferruginous cherts of the Gunflint formation of southern Ontario were carried out by electron microscopy in collaboration with Dr. Edward Anders and Dr. Fitch. Dr. S. A. Tyler of the University of Wisconsin and Dr. Barghoorn of Harvard University have reported (Science, Vol. 118, p. 606, 1954) the occurence of primitive lower plants in these Pre-cambrian rocks, which are the oldest (about two billion years) structurally preserved organisms that clearly exhibit cellular differentiation. Electron microscopy reveals the presence of filaments, tubular structures and membranes of apparent organic origin.

These studies are of great interest in the evolutionary scheme of primitive life, since they may furnish insight into the molecular organization of the oldest known preserved living systems bearing also on the evolution of membrane ultrastructure.

C. High Resolution Electron Microscopy with Superconducting Lenses at Liquid Helium Temperatures.

Following our first successful electron microscopy experiments with high field superconducting lenses operating at liquid helium temperature, a comprehensive research program has continued with different types of cryo-electron microscopes concentrating on two approaches:

1. Cryo-electron microscope optical bench system using high field superconducting niobium-zirconium solenoid lenses in liquid helium Dewars, operating at 4 to 32 kG without pole pieces, and with modified objective and objective-projector pole pieces. An additional current vernier control circuit for the superconducting objective solenoid is used in conjunction with a 25 A regulated power supply to permit adjustable current changes of 10-9 for achieving reproducible "superfine" focusing, orders of magnitude better than conventional systems. This device, specially developed for our lenses by D. Kasun and associates of Westinghouse Cryogenics Div., consists of a low field two-winding superconducting toroid with its secondary connected in series with the main solenoid. Energizing the toroid primary changes the total flux linkage and results in an incremental change of the field in the main solenoid.

   After optimum focusing adjustment is achieved, transfer of toroid primary to persistent mode places the entire system in a lossless, completely stable condition. Improved point cathode sources and highly stabilized 50 kV accelerating potential were used, taking special precautions to minimize mechanical, magnetic and electrical field perturbations. Once correct focus of test specimens at electron optical magnifications of 200x-20,000x is attained, the superconducting solenoid system is switched into persisten current mode.

   The high quality images (50Å-100Å resolution) thus maintained without any external lens current source are of an unprecedented degree of stability,

permitting exposures of up to several minutes with low intensity illumination for direct photographic recording on high resolution films. The same area can be continuously recorded at 5-15 minute intervals over a 10-hour period under carefully controlled conditions without detectable image changes, demonstrating typical long-term super-stability. Combination of this unique stability of superconducting lenses with coherent microbeam illumination appears promising for practical realization of Gabor's wavefront reconstruction microscopy.

2. Special superconducting objective lens in liquid helium cryostat which may be used as integral part of cryo-electron microscopes or replace the objective lens in modified high resolution commercial electron microscopes. The superconducting objective lens in a special Dewar, designed and built to our specifications by Westinghouse, comprises a main Nb-Zr coil (27,220 ampere-turns) with vernier coil, superconducting stigmators, persistent current switches and improved current control devices.

Specimens are mounted on microstages of special design maintained at $4.2^{\circ}K$ together with pole pieces of different types including short focal length, single field condenser objective pole pieces of iron or dysprosium, and trapped-flux Nb_3Sn lenses. Results of this most recent work are described in Proceedings of the National Academy of Science (1966), 56: 801-808.

It has now been possible to record, for the first time, electron micrographs of biological specimens at $4.2^{\circ}K$. Resolutions of 10 - 20Å were reproducibly attained, particularly in specimens of catalase crystals embedded in thin layers of heavy metals. These exhibited exceptional contrast and anomalous transparency phenomena.

The unique combination of high magnetic fields, liquid helium temperatures and high electron optical magnifications has enabled us to make preliminary observations on characteristic electron optical phenomena associated with trapped fluxes in thin superconducting films.

All of the results described above were presented in a keynote address before the Sixth International Congress for Electron Microscopy at Kyoto, Japan, August 28 to September 4, 1966. International evaluation, as expressed in particular by Professor Dupouy of the Laboratoire d'Optique du C.N.R.S. at Toulouse, was that our pioneering work has clearly established the value of superconducting solenoid lenses for electron microscopy, especially because of their superstability, the unprecedented high fields, and the possibility of now being able to consider other types of magnetic field configuration for obtaining much higher performance in high resolution electron microscopes.

Prof. Dupouy claimed as a noteworthy achievement the fact that since the first successful imaging experiments were carried out, barely 2 1/2 years ago, we have been able to implement a comprehensive research program, including the development and testing of many difficult components and solving other related methodological and conceptual problems.

Our work has been confirmed by Dr. Laberrigue, College de France in Paris, who stated that a new chapter has been opened up in electron optics with superconducting lenses which should prove to be of key significance for high voltage electron microscopy.

D. Development of improved instrumentation and preparation techniques has continued. Several important new results are described here for the first time.

1. Extending earlier work, improved point cathode sources have been developed and used routinely in high resolution microscopes. Pointed filaments are made of etched, oriented single-crystal tungsten tips (ca. 100μ long; tip radii; 0.1-10μ), spot-welded on zone-refined tungsten wire.

Because of high purity and selected crystal orientation these filaments have longer average life (40-80 hours at 2650°K; AC or DC heating) and operational stability than standard or by energetic ions commonly originating from impurities in normal tungsten sources. Similar pointed filaments of high purity tantalum, rhenium and niobium have specific properties particularly useful for low-temperature microscopy. Best results were consistently obtained with new types of etched molybdenum or stainless steel caps, the key element of which is a replaceable disc aperture of thin (10-30μ) molybdenum foil with a precisely centered small hole of 0.5mm.

The Mb foil is stable under electron bombardment which retards contamination and prolongs operational life of filament tips. Thin film configurations permit critical centering and height adjustment of filament tips determining adequate bias voltage and emission control. This practical cap design is applicable to both cathode and anode structures, combining optimum microgeometry and operational conditions for obtaining space-charge-free emission and significant field enhancement.

When used with compensated double condenser systems of high resolution instruments, these point sources provide improved micro-beam illumination characterized mainly by: (a) High specific brightness of 5 - 25 x 10^5 A cm^{-2} $sterad^{-1}$ measured reproducibly at final screen level. This gain in brightness is an order of magnitude larger than comparable values obtained with W hairpin filaments in standard guns at the

same temperature (2680°K). Mb thin-film caps give highest values, surpassing the performance of a special steel cap with a 0.5mm hole which was developed in collaboration with Dr. K. Yada as described in another publication. This high brightness is partly ascribed to field enhancement by the Schottky effect.

(b) High coherence, resulting partly from the smaller illumination angles made possible by increased source brightness. Transverse coherence lengths of 60 to 600Å can be achieved by using condenser apertures of 100μ-10μ. Under favorable conditions 50 to 60 Fresnel fringes were recorded. This also gives enhanced contrast and improved image quality, especially when dealing with biological specimens.

2. Using short focal length objective lenses (f = 1.8mm) of improved stability (circuitry for Elmiskop developed by H. Armbruster) we confirmed and extended the results of R. Heidenreich by recording phase contrast high resolution images of carbon atom arrays (hexagonal cells of 5Å) in ultrathin (10-20Å) single-crystal graphite specially prepared by a technique described earlier. Other pauciatomic structural patterns of organic systems were also resolved under similar conditions.

3. High resolution phase contrast was also achieved by precise alignment of multi-hole or annular condenser apertures and objective phase plates of composite ultrathin single-crystal films (graphite, mica silver) with adjustable electrical fields, or ferromagnetic thin-film apertures permitting phase shift control. In preliminary experiments, marked enhancement and resolution was noted in thin biological specimens. It was also possible to resolve structures of 5-8Å in unstained ferritin and apoferritin molecules.

4. Systematic use was made of ultrathin carbon films (10-80Å) prepared by evaporation in ultrahigh vacuum (10^{-8} torr) on special supports. Even thinner lamellae (10Å) of single-crystal graphite and diamond have been used which are extremely smooth and stable under intense irradiation, and can be used for high resolution shadow casting of DNA with carbon. These single-crystal substrates may be ideal atomic

monolayer substrates for high resolution electron microscopy.

5. Biological specimens were mounted without background support on asbestos filaments or related substrates. DNA strands were also mounted on thin single-crystal mica with regular holes (80-100ÅØ) produced by fission tracks.

6. Extending earlier work, wet or hydrated biological specimens were examined by using special vacuum-tight microchambers, low intensity microbeam illumination, and cryogenic devices to minimize dessication and radiation damage.

7. Thin, frozen sections of native, unfixed tissues can also be examined directly after ultrathin sectioning (20-100Å) with a diamond knife in microtomes operating in liquid nitrogen or helium cryostats, and transferred without thawing to the low temperature electron microscope stage.

8. Useful resolutions of 6-15Å have been consistently attained. This corresponds in many cases to resolution of quaternary or tertiary structures in biological membranes and derivatives (1,2) multienzyme complexes (5,6), hemocyanin and apohemcyanin (1,2) catalase and RNA-polymerase (3) and DNA and RNA systems (6,12).

9. With the improved instrumentation and preparation techniques already described, we have recently been able to record high resolution pictures at 330,000X electron optical (subsequent photographic magnifications up to 30 million X) of suitably oriented Phthalocyanine specimens (metal-free, as well as copper and other heavy metal salts of Phthalocyanine). The individual Phthalocyanine molecules are resolved; their structural details agree quite well with the corresponding diagrams of atomic positions.

10. A monograph entitled, "Elements of Biomolecular Organization", is being prepared to fill a gap between existing monographs on cell fine structure and on molecular structure. An attempt is made, by means of the case history approach, to document

representative examples of the orderliness of cells and their derivatives. This structural "orderliness", which in many cases is of crystalline or paracrystalline character, is shown by electron microscopy to extend throughout all hierarchies of organization from the cell to the subcellular level and beyond this into the domain of macromolecular assemblies.

E. Continued studies on the subunit structure of membranes, including mitochondria, nerve membranes, and photoreceptors: retinal rod outer segments, multienzyme complexes, fraction-I protein, and RNA-polymerase.

1. Investigations of nerve membranes and related structures were carried out by high resolution electron microscopy, using improved preparation and instrumentation techniques, which have already proved successful in the analysis of virus fine structure and other biological systems. Increasing emphasis has been placed on the study of the detailed organization of the enzyme and multi-enzyme complexes intimately associated with cell membranes as the molecular componentry, ultimately responsible for the highly specific energy and information transduction functions.

2. Investigations of the subunit structure of myelin membranes showed that the layers of the myelin sheath tend to dissociate into granular or rod-shaped particles of about 60Å. Correlated electron microscopic and x-ray diffraction data also indicated the possibility of a regular organization within the plane of the layers, probably involving units of 60 to 80Å.

Special techniques were applied in preparing ultrathin, frozen sections of fresh tissue. They were cut with a special microtome and diamond knife in a cryostat at -30° to -180°C. The specimens were often examined directly without thawing by embedding in vitrified heavy metal layers. A liquid nitrogen cooling device was used in conjunction with appropriate low-temperature electron microscopy techniques.

Ultrathin frozen sections of fresh frog sciatic nerve, negatively stained with phosphotungstate, show characteristic repeating particulate units of about 50 to 60Å. These units, mainly localized in the intermediate layers and attached to the dense myelin layers, were consistently found in the negatively stained, unfixed specimens. Bearing in mind the inherent artifact possibilities, these and related methods are now being further investigated in an attempt to examine the fine structure of the myelin sheath under conditions closely approximating its native hydrated state.

3. Organization of Membranes in Photoreceptors. Electron microscopy has confirmed earlier polarized light studies which indicated that the rod outer segments of vertebrate photoreceptors consisted of thin, transversely arranged protein layers which alternate with longitudinally oriented layers of lipid molecules. The fine structure of the outer segment was shown by electron microscopy to be composed of several hundred unit disks about 150 to 200Å thick.

Improved low-temperature electron microscopy techniques revealed an intermediate layer between the dense layers and electron-dense structures within the layers which appear as globular subunits. These subunits look well organized in dark-adapted outer segments and may be related to the presence of the photopigment complexes, as suggested by data from polarized light analysis and experimental modifications. Since rhodopsin represents about 40% of the dry weight in frog retinal rod outer segments, it may be regarded as a principal structural component of the photoreceptor system.

We have used special preparation techniques and low-temperature electron microscopy to examine outer segments of the frog retinal rod. Fresh, ultrathin sections of light and dark-adapted segments were prepared without fixing and without embedding. The specimens were kept frozen or dried and were stained, negatively or positively, with buffered phosphotungstate, uranyl formate, and other electron-dense reagents. Additional structural detail was detected in these well-preserved preparations. The globular subunits (60 to 70Å), which are prominent in the dense and intermediate layers, appear to be built up of minute particles, about 10 to 15Å regularly arranged in clusters or rows. Symmetrical alignment of these dense particles is usually maintained, even in certain regions where the dense layers are split.

These preparation techniques, which combine the advantages of thin sectioning with the enhanced preservation of negative staining applied to native, unextracted specimens, are particularly suitable for correlated biochemical studies. Moreover, when used in combination with enzymological assays of isolated retinal outer segments it should

be possible to obtain essential data on the localization of the visual pigments, with associated lipoprotein components and specific enzyme systems.

4. Macromolecular Repeating Units of Structure and Function in Mitochondrial Membranes. A large body of evidence has accumulated during the past decade showing that the electron transfer system, the respiratory transformations, and certain other systems of the mitochondrion are built up from characteristic macromolecular complexes.

Following the first observations by Fernández-Morán, other investigators including D. Smith, D. Parsons, and W. Stoeckenius, have confirmed the existence of the repeating particles and underlying subunit organization of mitochondrial membranes using a wide variety of techniques, including the improved freeze-etching method applied by Moor and Muhlethaler.

Since this technique operates on native frozen specimens and does not involve the use of chemical fixatives or stains, the successful demonstration of globular repeating units in mitochondria and other membrane systems has effectively disposed of tentative suppositions that these characteristic units might be preparative artifacts. It is, therefore, now generally agreed by most workers that these mitochondrial subunits exist, although the observed structural details will depend on the preparation techniques and experimental conditions.

Although subsequent studies by Green and Perdue indicate that there may be multiple species of mitochondrial elementary particles, the basic concept of a macromolecular repeating unit of mitochondrial structure and function is now generally accepted. The elementary particle of the mitochondrion is believed to be a prototype of a class of functional particles or macromolecular assemblies found in association with membranes generally.

5. Subunit patterns of neuronal membranes. Investigation of the molecular organization of the synapse is of particular significance in view of the specialized molecular switching componentry assumed to mediate information storage, transfer and retrieval at these junctional regions.

Unfortunately, neuronal membranes have not yet yielded to correlated biochemical and electron microscopic studies of the type successfully applied to mitochondrial and chloroplast membranes. Difficulties in reliably isolating and preparing these labile structures without introducing serious artifacts impose serious limitations.

Confirming and extending our earlier work, certain submicroscopic nerve fibers were found in frog spinal cord usually attached to the thicker nerve fibers. These fibers are thin enough to display their entire structure in a single electron micrograph, appearing as thin ribbons with diameters ranging between 0.1μ and 1μ and reaching lengths up to 50μ to 100μ.

With improved techniques, new ultrastructural detail was revealed in these well-preserved, ultra-thin nerve membrane specimens. They exhibit a characteristic surface structure featuring in certain regions hexagonal arrays of closely packed elements 50 to 90Å in diameter. Various types of dense granular components are found attached to the membranes, particularly in junctional regions.

These techniques can undoubtedly be considerably refined, and it should eventually be possible to examine these ultrathin submicroscopic nerve fibers in vacuum-tight microchambers under conditions approaching the native hydrated state.

6. Pyruvate and α-Ketoglutarate dehydrogenation multienzyme complexes. Extending our earlier collaborative work with Dr. L. Reed and his associates of the University of Texas, further electron microscopic and biochemical studies were carried out on the pyruvate dehydrogenase complex (PDC) and the α-Ketoglutarate dehydrogenation complex of E. coli.

The macromolecular organization of the α-keto-glutarate dehydrogenase complex (KGDC) isolated from E. coli appears to be similar to that of the PDC. As shown by electron microscopy, the smaller polyhedrical particles, with a diameter of about 280Å feature a tetrad core surrounded by peripheral subunits, which are not clearly defined as in the PDC complexes.

Recent studies by L. Reed and his associates indicate that the macromolecular organization of the bacterial and mammalian PDC and KGDC multienzyme complexes are fundamentally similar, and may be governed by the principles of self-assembly which determine the functional organization of protein shells of regular viruses with icosahedral symmetry.

7. Organization of L-glutamate dehydrogenase. Recent investigations of bovine L-glutamate dehydrogenase crystals, using micro-droplet cross-spraying techniques and microbeam illumination for high resolution electron microscopy, have made it possible to observe the ordered aggregation and fine structure of the relatively well preserved molecules in the thin microcrystals.

Individual spherical or polyhedral particles of 130Å to 150Å diameter can be frequently observed, either free or closely packed in ordered aggregates of the thin crystalline strands. The individual polyhedral particles of 130Å to 150Å may correspond to the undissociated individual molecules, while the numerous subunits of triangular shape could be partly related to the GDH tetrahedral subunits described by Horne and Grevel. However, on the basis of available data, the organization of the undissociated molecules appears to be more complex than the suggested simple aggregate of four subunits. Further studies are being pursued in attempts to elucidate the detailed subunit structure of these enzyme molecules.

8. Fraction-I Protein. In collaboration with R. Haselkorn, F. Kieras and associates, work has continued on F-I protein from Chinese cabbage leaves and other sources, including blue green algae. This work, which is still in progress, has confirmed our original observations (as reported in Science (1965), 150: 1598-1601. The F-I protein is seen to consist of uniform cubical particles with an edge of about 120Å.

In high resolution electron micrographs of negatively stained preparations the cubical particles exhibit a characteristic subunit structure which is consistent with a model having 24 subunits, in agreement with the present physical and chemical data.

The fact that the substructure of a protein enzyme of only 120Å can now be directly observed in electron micrographs, is of particular relevance to investigation of the enzyme complexes of similar size, which are presumably associated with nerve membranes and derivatives.

9. RNA-polymerase. Correlated electron microscopic and biochemical studies have continued on the organization of a DNA-dependent RNA-polymerase from E. coli. Related studies on the association of RNA-polymerase with DNA from Ø x 172 are being carried out in collaboration with Dr. Samuel Weiss, University of Chicago. These investigations are expected to yield further information on RNA-polymerase and its participation in the differential RNA transcription upon DNA templates, which is of fundamental importance in the regulation of protein synthesis and function in cell membranes and their derivatives.

II. Organization and Operation of the Special Electron Microscope Laboratories in the Department of Biophysics, The University of Chicago.

With funds provided by The University of Chicago, NASA Grant (NsG 441-63), NIH Grants (B-2460, NB-04267, GM-13243), and AEC Grants (AT 30-1-2278, AT 11-1 1344), a special laboratory facility for high resolution electron microscopy has been completed and put into operation in the Research Institutes. These laboratories occupy a total of about 4,000 square feet and comprise:

A. 2,500 square feet of remodeled space in the basement with installation of special floors, wall partitions, ceiling panels, air conditioning of the type used in "clean rooms" for modern electronic industrial facilities. These laboratories are equipped with three electron microscopes with attached electron diffraction units. The facilities include ultra-high vacuum (Varian) evaporation units, four ultramicrotomes, light microscopes, and complete preparation and photographic darkroom facilities. All of the critical equipment has been installed on individual vibration control mountings of special design. Corresponding precautions were taken in the installation of non-magnetic stainless steel ventilation ducts, incandescent lights, and electrical conduit to minimize electrical and magnetic perturbations.

B. Adjoining laboratories of 920 square feet located on the second floor of the Research Institutes have been remodeled:

1. Room 203B (230 sq. ft.)

This room has been prepared for storage of specimens, equipment, and laboratory apparatus. An Harris Cascade Refrigeration Biological Storage Machine was installed which operates at temperatures as low as -120°C.

2. Room 205 (230 sq. ft.)

This room has been prepared as a site for superconducting experiments. A Siemens Elmiskop EM-II with accessories was installed. Darkroom equipment has also been installed to expedite development of plates taken during experiments in this room.

3. Room 207 (460 sq. ft.)

An X-ray diffraction unit with Kratky Camera and special liquid helium specimen cooling device was installed.

Two vacuum pumping units were developed and installed which pre-pump photographic plates (at a rate of 912 plates per 2 hours), also with capacity to pre-pump 70mm film and camera. The efficiency of these pumps is such that it reduces working time by several hours. Plates, film, and camera were previously pre-pumped in the microscope itself which involves a much longer time.

C. An additional laboratory (Room P-III) was constructed in the clean room laboratories of the basement to house a Hitachi Perkin Elmer electron microscope and accessories. This microscope was installed and includes a double condenser lens, electron diffraction chamber, hot and cold stages, and image intensification system.

D. A highly regulated power supply is located in an air conditioned enclosure on the fifth floor of the Research Institutes. This 50-kilowatt motor generator set, specially designed and manufactured by Westinghouse Company, is equipped with a new solid-state regulator, giving better than 0.1% voltage stability and very low harmonic distortion.

E. A vibration-free room was built for installation of the new "cryo-electron microscope," adapted from the Hitachi 11-B electron microscope purchased with NASA funds. The ten-ton floating foundation and other features of this facility should make it possible to exploit the unique stability of superconducting lenses operating in the persistent current mode for long-term exposures: of the order of minutes to hours, instead of the 5 to 15-second exposures presently possible.

III. Training Program.

In addition to a specific research program, the laboratory has served as a center for information exchange to investigators and students in the United States and other countries.

1. A course in Cell Ultrastructure (Biophysics 308) was given as part of the Biophysics Curriculum. The enrollment (30 participants) included students and faculty from the Biophysics, Microbiology, Zoology, Botany, Pathology and Medicine departments of the University of Chicago, in addition to visitors from the Downey Veterans Administration Hospital.

2. Participation in the following conferences has also brought about considerable information exchange:

   a. High Voltage Electron Microscope Workshop at Argonne National Laboratory, June 13 to July 15, 1966.

      1. Lecture on Superconducting Lenses - June 14
      2. Chairman, Summary Session - July 14

   b. The Neurosciences Research Program Intensive Study Session at Boulder, Colorado, July 17 to August 12, 1966.

      1. Lecture on "Membrane Ultrastructure in Nerve Cells" - July 27

   c. Electron Microscopy Society of America in San Francisco, August 22 to 25, 1966.

      1. Lecture as keynote speaker on "New Approaches in High Resolution Electron Microscopy."

   d. Sixth International Congress for Electron Microscopy at Kyoto, Japan, August 28 to September 4, 1966.

      1. Keynote address on "High Resolution Electron Microscopy of Biological Specimens," Opening Session, August 28.

2. Lecture on "Application of Improved Point Cathode Sources to High Resolution Electron Microscopy," August 29.

3. Lecture on "Low Temperature Electron Microscopy with High Field Superconducting Lenses," September 1.

4. Chairman of afternoon session, August 29, on High Resolution and Lens Aberrations.

e. Department of Anatomy, guest lecture, University of Chicago, December 12, 1966.

f. Fifteenth Annual Edsel B. Ford Memorial Lecture, January 17, 1967, Edsel B. Ford Institute for Medical Research, Detroit, Michigan, entitled "Seeing, and Design in the Molecular Domain."

IV.A. List of Publications for the Period April 1, 1966 through December 31, 1966 (5 copies of each are included with this report).

1. H. Fernández-Morán, E.F.J. van Bruggen, and M. Ohtsuki, Macromolecular Organization of Hemocyanins and Apohemocyanins as Revealed by Electron Microscopy, J. Mol. Biol. (1966), 16: 191-207.

2. E.F.J. van Bruggen and H. Fernández-Morán, Re-association of Hemocyanins from Subunit Mixtures, J. Mol. Biol. (1966), 16: 208-211.

3. A. J. Colvill, E.F.J. van Bruggen, and H. Fernández-Morán, Physical Properties of a DNA-dependent RNA Polymerase from Escherichia coli, J. Mol. Biol. (1966), 17: 302-304.

4. H. Fernández-Morán, Forms of Water in Biologic Systems and the Organization of Membranes, in Annals of the New York Academy of Sciences (1965), 125, Art. 2, 739.

5. R. Haselkorn, H. Fernández-Morán, F.J. Kieras, E.F.J. van Bruggen, Electron Microscopic and Biochemical Characterization of Fraction I Protein, Science (1965), 150: 1598-1601.

6. H. Fernández-Morán, High Resolution Electron Microscopy of Biological Specimens, Proc. Sixth International Congress for Electron Microscopy, Kyoto (1966), Vol. I, 13.

7. H. Fernández-Morán, Applications of Improved Point Cathode Sources to High Resolution Electron Microscopy, Proc. Sixth International Congress for Electron Microscopy, Kyoto (1966) Vol. I, 27.

8. H. Fernández-Morán, Low Temperature Electron Microscopy with High Field Superconducting Lenses, Proc. Sixth International Congress for Electron Microscopy, Kyoto (1966), Vol. I, 147.

9. H. Fernández-Morán, Microscope, Electron, McGraw-Hill Yearbook of Science and Technology (1966), 249-252.

10. H. Fernández-Morán, High-Resolution Electron Microscopy with Superconducting Lenses, Proc. AMU-ANL Workshop of High Voltage Electron Microscopy, pp. 51-58, presented at the Workshop for High Voltage Electron Microscopy, Argonne National Laboratory, June 13-July 15, 1966.

11. H. Fernández-Morán, High-Resolution Electron Microscopy with Superconducting Lenses at Liquid Helium Temperatures, Proc. Nat'l. Acad. Sci. (1966), 56: 801-808.

12. H. Fernández-Morán, Potential Application of Electron-Optical Methods to Storage of Information for Direct Retrieval, in Biology and the Exploration of Mars, National Academy of Sciences--National Research Council, Publication No. 1296, Washington, D.C., 1966; pp. 503-506.

13. H. Fernández-Morán, Low Temperature Electron Microscopy with High Field, Superconducting Lenses. Abstract of paper presented at National Superconductivity Information Meeting, Brookhaven National Laboratory, Nov. 9-11, 1966.

14. H. Fernández-Morán, Membrane Ultrastructure in Nerve Cells, to be published in The Neurosciences, A Survey for Synthesis, based on the NRP Summer, 1966 Intensive Study Program in the Neurosciences, edited by F. O. Schmitt and T. Melnechuck, Rockefeller University Press.

15. a. H. Fernández-Morán, Pointed Cathode Sources for High Resolution Electron Microscopy, Part I.

    b. K. Yada and H. Fernández-Morán, Pointed Cathode Sources for High Resolution Electron Microscopy, Part II, abstract of paper to be submitted to the Journal of Applied Physics.

    c. K. Yada and H. Fernández-Morán, Pointed Cathode Sources for High Resolution Electron Microscopy, Part III, abstract of paper to be submitted to the Journal of Applied Physics.

16. H. Fernández-Morán, Elements of Biomolecular Organization, An Atlas of Biological Ultrastructure, in press, to be published by Springer-Verlag, Berlin, Heidelberg, New York.

B. List of Supporting References (5 copies of each are submitted with this report).

1. "Three New Designs Improve Electron Microscopes," article in Scientific Research, January, 1966.

2. "Voltage, Lenses Aid Electron Microscopy," article in Chemical Engineering News, September 26, 1966.

3. "Superconducting Lens Improves Electron Microscope's Resolution," article in Dateline in Science (December 2, 1966), Vol. 1, No. 3, p. 1.

4. Reference to high resolution electron microscopy of graphite structure published in Science (1966), 154: 195.